(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 174 810 B1**

B2

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication of patent specification: **21.07.93**

(51) Int. Cl.5: **C12N 15/12**, C12N 1/20, C12P 21/00, //(C12N1/20, C12R1:19)

(21) Application number: **85306331.1**

(22) Date of filing: **05.09.85**

The file contains technical information submitted after the application was filed and not included in this specification

(54) Multidrug resistance in mammalian cell lines and isolation of determinant glycoprotein DNA.

(30) Priority: **10.09.84 GB 8422819**
03.09.85 CA 489897

(43) Date of publication of application:
19.03.86 Bulletin 86/12

(45) Publication of the grant of the patent:
21.07.93 Bulletin 93/29

(84) Designated Contracting States:
CH DE FR GB IT LI NL SE

(56) References cited:

NATURE, vol. 309, no. 5969, 14th June 1984, pages 626-628, London, GB; I.B. RONINSON et al.: "Amplification of specific DNA sequences correlates with multi-drug resistance in Chinese hamster cells"

SCIENCE, vol. 221, 23rd September 1983, pages 1285-1288, Lancaster, US; N. KARTNER et al.: "Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines"

(73) Proprietor: **HSC Research Development Corporation**
88 Elm Street
Toronto Ontario, M5G 1X8(CA)

(72) Inventor: **Riordan, John R.**
137 Bedford Road
Toronto M5R 2K7 Ontario(CA)

(74) Representative: **Adams, William Gordon et al**
RAWORTH, MOSS & COOK 36 Sydenham Road
Croydon Surrey CR0 2EF (GB)

EP 0 174 810 B1

CHEMICAL ABSTRACTS, vol. 101, no. 7, 13th August 1984, page 145, abstract no. 49442p, Columbus, Ohio, US; V. LING et al.: "DNA-mediated transfer of multidrug resistance and expression of P-glycoprotein", & PROG. CANCER RES. THER. 1984, 30(GENE TRANSFER CANCER), 53-7	
NATURE, vol. 316, no. 6031, 29th August 1985, pages 817-819, London, GB; J.R. RIORDAN et al.: "Amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines"	

Description

This invention relates to living eucaryotic cells developing resistance to drugs which are normally toxic to them and more particularly, to the determinant of drug resistance of living cells and the nucleic acid sequence encoding for the protein determinant.

It is known that mammalian cells develop resistance to drugs, such as discovered in the field of cancer research where cancerous cells develop resistance to drugs used in chemotherapy. This resistance halts the effectiveness of the drugs used in chemotherapy for slowing down or ceasing the multiplication of the cancerous cells. Recently reported in Science, Vol. 221, pp 1285-1288, September 1983, reference is made to hamster, mouse and human tumorous cell lines displaying multiple drug resistance. It was discovered that there is an increased expression of a 170,000-dalton surface antigen correlated with multidrug resistance as found in the cell membrane. The antigen has been isolated and is understood to be a P-glycoprotein.

Multidrug resistance (MDR) is the phenotype exhibited by mammalian cell mutants particularly selected for resistance to a certain anti-cancer drug, but also exhibiting resistance to a broad spectrum of other cancer drugs having different chemical structures and targets of action. Cells with this phenotype maintain reduced intracellular levels of drugs as the apparent mechanism of resistance. Related to this altered drug transport function, the plasma membranes of these cells contain elevated amounts of the polypeptide specifically referred to as P-glycoprotein.

A publication by Roninson I.B. et al (1984) Nature, Vol. 309, pp 626-628 describes a cloned DNA sequence, i.e. a 170 KD glycoprotein, which is associated with multidrug-resistance in CHO cells. However this sequence is not the P-glycoprotein of the present invention.

It has been possible to select a variety of mutant cell lines with different degrees of drug resistance. The amount of P-glycoprotein in the plasma membranes of these different cell lines correlates quantitatively with the degree of drug resistance.

Over-expression of P-glycoprotein appears to be a consistent and characteristic feature of the multidrug resistance phenotype.

Extensive work has been conducted to demonstrate this P-glycoprotein directly or indirectly mediates the multidrug resistance phenotype. Extensive genetic studi s hav been carried out on Chinese hamster ovary cell systems as disclosed in Ling V., Kartner, N., Sudo, T., Siminovitch, L. and Riordan, J.R. Cancer Treat. Rep. 67, 869-874

(a) independent drug resistant clones isolated in the single step without mutagenesis display the multidrug resistanc phenotype and P-glycoprotein over-expression;

(b) selection for increased drug resistance, i.e., colchicine resulted in increased cross-resistance to other drugs and increased P-glycoprotein expression;

(c) revertants isolated in a single step for drug sensitivity to one compound involved in the multidrug resistance phenotype, displays reversion of the other aspects of the phenotype including reduced P-glycoprotein expression;

(d) cross-resistance, collateral sensitivity, and P-glycoprotein over-expression are concordantly expressed in cell:cell hybrids.

A publication by Riordan, J. R. et al (29.08.85), Nature, Vol. 326, pp 817-819 published after the priority date of the present Application describes the amplification of P-glycoprotein genes in multidrug-resistant mammalian cell lines and showed that P-glycoprotein is conserved and is probably encoded by a gene family.

The unknown aspect with respect to the isolation of the P-glycoprotein and its determinant in causing multidrug resistance is whether or not a protein of this size is coded by a single gene or family of genes or the result of independent events in the selective expression of a gene or gene family.

According to the invention there is provided a purified DNA molecule of approximately 600 bp characterized in that said DNA molecule codes for a polypeptide portion of approximately 200 amino acids of a P-glycoprotein which has a total molecular weight of approximately 170,000 daltons and an isoelectric point of 7.4, said DNA molecule including DNA molecules pCHp-1 and pCHp-2 having respectively ATCC accessions numbers 39839 and 53234, said P-glycoprotein being a major determinant in multidrug resistance of living cells.

The purified DNA molecule may be constructed of cDNA, mRNA or a DNA molecule which codes for a polypeptide portion of approximately 200 amino acids of the P-glycoprotein and may be incorporated in recombinant form with a bacterial phage or plasmid. The phage or plasmid may be cloned and when transformed in a suitable host cell, th host cell may be cultured to produce polypeptide portions of approximately 200 amino acids of the P-glycoprotein. The DNA molecule in the form of a bacterial phage and/or plasmid may be used as a DNA probe with suitable marker provided on the phage or plasmid.

Th invention also provides a plasmid comprising th DNA molecule as pr viously described in recombinant form with said plasmid.

The present invention further provides a host cell comprising a plasmid as previously described and a microorganism comprising a plasmid as previously described.

The present invention still further provides a method of producing a polypeptide portion of a P-glycoprotein having a total molecular weight of approximately 170,000 daltons characterised by culturing a microorganism in an aqueous nutrient medium and isolating said polypeptide.

According to the invention there is further provided a method for isolating in a plasmid the purified DNA molecule in the form of cDNA wherein a cDNA library is constructed using a known bacterial phage expression vector and mRNA obtained from highly drug-resistant cell lines which overexpress the P-glycoprotein. By appropriate techniques, such as the use of monoclonal antibodies, the phage vectors which express a fusion protein antigenic to an antibody, which is specific to the P-glycoprotein, are identified. A recombinant plasmid is formed from the identified phages. The plasmid is expressed in a compatible living cell to produce a polypeptide portion. The antibody is used to confirm that the polypeptide is a portion of the P-glycoprotein.

Preferred embodiments of the invention are discussed with respect to the drawings, wherein:

Figure 1 is the identification and isolation of a cDNA sequence corresponding to a portion of the P-glycoprotein, employing three monoclonal antibodies. Panel A shows the stained fusion protein with a molecular size of 140,000 daltons. Panel B shows the reactivity of this fusion protein with the monoclonal Ab(C219) used to identify the clone and two other monoclonals specific for P-glycoprotein;

Figure 2a is Northern blot analysis of total cellular RNA from drug sensistive flux B1 cells and highly multidrug resistant B30 cells, the third lane containing RNA from normal bovine brain serves as a control;

Figure 2b is Slot blot analysis of RNA from a series of cells with varying levels of drug resistance similar to those in Figure 1, the relative degrees of resistance being indicated opposite each slot;

Figur 3a is a Southern blot analysis of EcoR1 digested genomic DNA from the same series of increasingly multidrug-resistant CHO cells as in Figure 2a;

Figur 3b is a Southern blot analysis of EcoR1 digested DNA from drug sensitive human leukemic cell line (CEM) and a multidrug resistant variant (CEM/Vlb1000). The other pair ar from drug sensitive mouse L cells (LMTK$^-$) and MDR variant (ECH^R). Methods wer as in Figure

Figures 4a, b and c show cytological evidence for localization of P-glycoprotein sequences to a homogeneously staining region on the Z4 chromosome of CHO cell line B-30.

Methods have been developed for the isolation and characterization of plasma membrane vesicles which serve as the starting material for the purification of P-glycoprotein. The protein has been purified and characterized as an intrinsic glycoprotein having a polypeptide moiety of a molecular weight of 140 kilodaltons and as a corresponding P-glycoprotein having a molecular weight of 170 kilodaltons and an isoelectric point of about 7.4. Polyclonal and monoclonal antibodies to the protein have been raised.

The developed monoclonal antibodies specific for and which strongly cross-react with the glycoprotein from different species are used to screen living cells which include the glycoprotein as part of the membrane. A complementary DNA (cDNA) library may be constructed from messenger RNA (mRNA) from the highly drug resistant cell line which overexpresses the glycoprotein. The library may be constructed by using the expression vector λgt11. Phage plaques expressing β-galactosidase fusion proteins antigenic to the monoclonal antibody specific for P-glycoprotein are identified, picked and analyzed in an attempt to locate a cDNA encoding for a polypeptide sequence of the P-glycoprotein.

Membrane proteins from different multidrug resistant lines have been immunoblotted with polyclonal antibodies specific for P-glycoprotein as disclosed in Science Vol. 221 supra. The degree of P-glycoprotein expression has been found to correlate with the relative drug resistance of the cell lines. Thus, the over-expression of P-glycoprotein appears to be the most consistent molecular marker associated with the multidrug resistance phenotype for living cells. It has been found that there is over-expression of P-glycoprotein in biopsy samples obtained directly from tumour cells of ovarian patients with advanced ovarian tumours which were non-responsive to chemotherapeutic treatment.

In order to isolate the nucleic acid sequence encoding for the polypeptide segment isolated by use of the monoclonal antibody, the complementary DNA library is constructed. One of the raised monoclonal antibodies (C219)to the P-glycoprotein is used to screen this library for clon s which synthesize β-galactosidase-P-glycoprotein fusion products. Several phag s were identified and plaque purified.

One of the presumptiv glycoprotein cDNA clones, now in the form of λ CHP-1 was isolated and subcloned in the plasmid pUC-9 to yield th

of approximately 600 base pairs which can be used as a probe for molecular hybridization analysis of nucleic acid sequences from drug resistant and drug sensitive cells. The insert of the plasmid pCHP-1 of approximately 600 base pairs encodes from the carboxyl end of the protein at least a portion of the P-glycoprotein which is over-expressed in the isolated drug resistant cell. In using the nucleic acid sequence as a probe for determining if cells are developing drug resistance, the nucleic acid sequence may be labelled with a detectable marker. The marker may be in the form of a label which is radioactive, fluorescent or biotinylated.

According to this invention, for purposes of cloning the plasmid pCHP-1, the plasmid may be transformed into a host microorganism for purposes of making multiple copies of the plasmid. The microorganism may be a strain of E. coli which acts as the host cell for the cloning vehicle. The polypeptide, which is a part of the P-glycoprotein as the determinant factor in cell resistance to drugs, may be produced by culturing the host microorganism or any other organism into which the cloning vehicle is transformed.

The host cell for the cloning vehicle, according to an embodiment of this invention, is the strain JM83 of E. coli K-12, Messing, J., Recombinant DNA Technical Bulletin, NIH Publication No. 79-99, 2, No. 2 (1979) 43-48. This host cell with the novel plasmid may be obtained through ATCC accession No. 39839 deposited on September 7, 1984. This host cell contains a recombinant plasmid, pCHP-1. This plasmid was constructed by insertion of a cDNA sequence corresponding to a portion of the coding sequence of the P-glycoprotein into the unique EcoR1 cloning site of the plasmid cloning vector, pUC9 (Vieira, J. and Messing, J., Gene, 19 (1982) 259-268).

The cDNA clone pCHP-1 codes for a C-terminal segment of P-glycoprotein consisting of approximately two hundred amino acid residues. The DNA insert in this clone is estimated to be about 650 bp in length. The fusion product consisting of the polypeptide coded for by at least a portion of this sequence plus β -galactosidase has a size of about 140,000 daltons. Subtraction of the β -galactosidase molecular weight of 116,600 daltons yields 23,400 daltons as the molecular weight of the peptide coded for by the insert sequence. This represents about 190 amino acid residu s or 570 bp. Therefore, all but about 80 bp of the insert are required to cod for the peptide. These 80 bp must represent a portion of the 3' untranslated sequence.

pCHP-1 was used to re-screen the same lamda library and five additional cDNA clones were obtained. The largest of these was approximately 2.5 kb. The longest which codes for approximately one half of complete P-glycoprotein polypeptid was designated pCHP-2 and deposited with ATCC on July 30th, 1985 and given accession No. 53234.

It is appreciated, however, that with extensive exacting procedures the nucleic acid sequence for the polypeptide portion of the P-glycoprotein is isolated by selecting mammalian cell lines which exhibit resistance to drugs, such as adriamycin, actinomycin D, colchicine, daunorubicin, emetine, podophyllotoxin, puromycin, taxol, vinblastine or vincristine, all of which frequently exhibit a pleiotrophic phenotype of multidrug resistance. This complex phenotype involves cross-resistance to structurally and functionally unrelated compounds. Although the plasma membrane of the cell is implicated in this complex phenotype of drug resistance by the cell, the actual mechanisms involved are not fully understood. According to an embodiment of the invention, the extensive procedures for isolating the nucleic acid sequence of the P-glycoprotein gene include selection of multidrug resistant mutant cells; their genetic characterization; drug transport studies; plasma membrane characterization including isolation of a P-glycoprotein to which antobidies are prepared. The antibodies are then used to screen a bacteria phage cDNA expression vector library prepared from the mRNA isolated from highly multidrug resistant mutants and the positive recombinant bacteria phage clones identified can then be used to subclone the P-glycoprotein specific DNA sequence into a plasmid vector. In this manner, the entire P-glycoprotein gene can be isolated and characterized.

It is appreciated that a segment isolated and cloned in plasmid pCHP-1 may include encoding regions which are not operable in coding for the polypeptide segment of the P-glycoprotein. It is believed that the operative portion of the nucleic acid insert has approximately 600 base pairs of the approximately 650 base pairs which may be in the recombinant plasmid pCHP-1. As established and shown in Figure 1, the protein moiety of P-glycoprotein is about 140,000 daltons which would require a messenger RNA with a coding region of about 4 kb. In cells with over-expression of the P-glycorotein, the single major mRNA component is of about 5 kb. The difference in weight is du to th 5' and 3' untranslated regions of the mRNA which would increas the siz of the P-glycoprotein mRNA to about 5 kb.

The polypeptide determinant for drug cell resistance is identified as a P-glycoprotein having a molecular weight in the range of 170,000 daltons. The concentration of the cell surface glycoprotein is quantitatively correlated with the multidrug resis-

variety of conditions. Highly resistant lines have increased expression of P-glycoprotein in the cell membrane. This membrane component appears to be overexpressed in other multidrug resistant lines isolated in animal and human cells. At least part of the P-glycoprotein molecule is highly conserved; i.e., constant among P-glycoprotein in different species. The monoclonal antibodies raised strongly cross-react with P-glycoproteins from different animal and human cells.

The monoclonal antibody (C219), which was used to identify λgt11 clones containing P-glycoprotein sequences, was raised using isolated plasma membrane vesicles, which were prepared as described in Riordan, J.R. and Ling, V. (1979) "Purification of P-Glycoprotein from Plasma Membrane Vesicles of Chinese Hamster Ovary Cell Mutants with Reduced Colchine Permeability", J. Biol. Chem, 254: 12701-12705, from highly colchine resistant CHO cell line (B30) as immunogen. Mice were immunized with these vesicles. After several booster injections, cells from the spleens of the mice were fused with a myeloma cell line to produce a battery of hybridoma cells. These were screened for the production of antibodies to the P-glycoprotein vesicles of multidrug resistant cells, but not those from drug sensitive cells.

Cell lines of increased drug resistance have increased P-glycoprotein expression. Figure 2b shows Slot blots of mRNA prepared from a related series of CH^R clones with increasing drug resistance, AuxB1 → CH^RA3 → CH^RB3 → CH^RC5 →→→ CH^RB30 as well as the revertant clone I10 which was selected from CH^RC5. The amount of mRNA revealed by the pCHP-1 probe in these cell lines is consistent with the levels expected if pCHP-1 sequences encoded a portion of P-glycoprotein.

Southern blot analysis of genomic DNA from the drug sensitive and series of related multidrug-resistant CH^RCHO cell lines of Figure 2b is shown in Figure 3a. It is clear that sequences homologous to pCHP-1 are amplified in the resistant lines compared with the drug-sensitive or revertant lines. Moreover, the amount of amplification correlates with increased drug resistance. This indicates that over-expression of P-glycoprotein in these lines resulted from gene amplification. There appears to be ten EcoR1 fragments ranging in siz from 1 to 15 kb homologous to PCHP-1 in the genome of the drug-sensitive cell lines. Multiple bands are also observed when other restriction enzymes w re used (data not included in Figur 3a). Multiple bands may result if the pCHP-1 prob were to span more than one exon; however, since the pCHP-1 probe does not have intemal EcoR1 sites, and sinc the insert size is only about 600 - 700 bp, the multiple bands observed in Figur s 3a and of genes coding for corresponding P-glycoproteins all of approximately the same molecular weight. The P-glycoprotein gene family appears to be clustered within one amplifiable unit (an amplicon). This is supported by the observation that in the clonal line selected for resistance to colchicine in a single discrete step, e.g. CH^RA3 of Figure 2b, simultaneous amplification of the ten restriction fragments occurred. Moreover, each fragment is amplified nearly equally by about 10-20 fold when compared with the equivalent fragment in the drug-sensitive line. In other systems, amplicons have been estimated to be approximately 100 to 1,000 kbp in size, thus the P-glycoprotein gene family is believed to be organized in a tandem array within such a region. A similar co-ordinant increase in amplification of P-glycoprotein genes is not observed in the second-step clone (CH^RB3), the third-step clone (CH^RC5), or the multistep line (CH^RB30). The restriction pattern becomes more complex, and the degree of amplification among the different restriction fragments appears to vary to a greater extend. In CH^RB30 for example, restriction fragments 4, 6, and 9 were amplified by 10-20 fold, while fragments 7, 10, 11, 12 were amplified 50 fold or more as shown in Figure 3a. These numbers were estimated by quantitative densitometry of appropriately exposed films in a separate experiment. Of interest was the appearance of amplified fragments in the more resistant lines which were not represented in the parental DNA. Examples of these are fragments 2 and 5 of Figure 3a. These may represent "novel joint" regions found in tandemly amplified sequences as described in Stark, F.R. and Wahl, G.M. Ann. Rev. Biochem. 53, 447-491 (1984).

The above observations indicate the CHO cell genome contains a family of as many as ten P-glycoprotein genes which are linked, and that this gene family is amplified in colchicine-resistant, multidrug-resistant cell lines. Southern blot analysis of multidrug-resistant mouse and human lines using pCHP-1 demonstrated that P-glycoprotein sequences in these species are also encoded by a family of genes and that some members of the family are amplified in the resistant lines as shown in Figure 3b. Eight EcoR1 fragments containing distinct P-glycoprotein gene sequences of the family are observed in the mouse genome. Five of these fragments ar clearly amplified in the resistant line; however, the other thr e fragments migrating just below the 9.6 kb and 4.3 kb mark r, and just above the 2.3 kb marker, ar apparently not amplified. Similarly, in th human genome eight fragments are observed but only four of the eight fragments are clearly amplified in the resistant line. The finding that som of the EcoR1 fragments are

resistant cell lines suggests that the P-glycoprotein genes in these lines may not be contained in a single amplicon and may be dispersed, in contrast to what was observed in CHO cells. In the resistant mouse cells a new fragment of about 3 kb not present in sensitive cells is observed as shown in Figure 3b. This is consistent with a "novel joint" region in the tandemly amplified sequences as mentioned above. Such a fragment is not observed in the resistant human line. The fact that pCHP-1 is able to hybridize strongly to the presumptive human P-glycoprotein sequences under stringent conditions provides further confirmation that P-glycoprotein is conserved both at the protein and DNA level.

Karyotypic features of gene amplification observed in a number of systems are chromosomal homogeneously straining regions (HSRs) or the presence of double minute chromosomes (DMs) observed in metaphase cells. Such features are known in multidrug-resistant cell lines. In DNA-mediated transfectant cells, the number of DMs correlates with colchicine and multidrug resistance, and the degree of expression of P-glycoprotein. In the CHO cell system, an extensive HSR is observed in CH^RB30 cells as shown in Figure 4, which is not found in the drug-sensitive parental cell line. This region is variable in length, occasionally being almost the size of the Z4 chromosome on which it is located. In order to determine if the amplified P-glycoprotein sequences in CH^RB30 cells were contained within this HSR, in situ hybridization was performed using ^3H-labelled pCHP-1 probe. Of 25 cells examined, 20 yielded one or more autoradiographic grains localized to the HSR of chromosome Z4 as shown in Figures 4b and 4c. When total chromosomal grains are scored, 45% (60/133) of all grains are localized to the HSR. In contrast, no significant grain localization was apparent in 25 cells examined using the drug-sensitive parental line AuxB1, the data for which is not shown in Figure 4.

Characterization of one or more of the genes of the gene family coding for the polypeptide of the P-glycoprotein is accomplished in accordance with the above discussed procedures using the isolated portions of cDNA. The DNA sequence for coding the polypeptid moiety of P-glycoprot in is of approximately 5 kb pairs. This DNA or any segment thereof can be used in a variety of ways to investigate multidrug resistance of cells. Furthermore, one or more genes of the family or a segment of any one of the genes of the family may be used as DNA probes to determine the xistenc of multidrug resistance in a living cell. Th gene or a segment thereof can be incorporated into a suitabl vehicle in the manner discussed and transformed or a portion thereof.

Preferred embodiments of the methodology in arriving at the invention will be exemplified in accordance with the following Examples.

EXAMPLE 1

Total cellular RNA was isolated from the highly colchicine resistant CHO cells, B30 according to Chirgwin et al BIOCHEMISTRY 18, 5294-5299 (1979). Poly A^+RNA was selected from this by chromatography on oligo dT-cellulose (Type 3, Collaborative Research) according to Aviv, H. and Leder, P., Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412 (1972). The product of this step was used as a template for the synthesis of cDNA employing oligo-dT as a primer. 1 µg of poly A^+RNA was heated to 70°C for 5 min., chilled on ice and then incubated for 1 hr. at 42°C in 50 mM Tris-HCl, pH 8.3, 40 mM KCl, 8mM $MgCl_2$, 0.4 mM DTT, 0.5 µg oligo dT (12-18; Collaborative Research), 2mM each deoxynucleotide, 1 µl of $\alpha[^{32}P]$dCTP (Amersham, 800 Ci/mmol) and 5 units of AMV reverse transcriptase (purified by Sephacryl S-200 chromatography). Second strand synthesis was performed at 14°C for 5 hr. in the presence of 100 mM HEPES, pH 6.9, 200 mM KCl, 0.2 mM of each deoxynucleotide and 2 µg of homogeneous DNA polymerase 1. Following Sephadex G-100 chromatography in 1 mM Tris-HCl, pH 7.5, 10 µM EDTA, the double stranded cDNA-containing fractions detected by Cherenkov radiation were pooled and lyophilized. The hairpin loop was digested with a 1000 U of S1 nuclease (Boehringer-Mannheim) for 1/2 hr. at 34°C. Following phenol/chloroform extraction the Sephadex G-100 chromatography step was repeated. Methylation at EcoR1 sites was performed after dissolving the pooled, lyophilized fractions in 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 10 µM S-adenosyl methionine. Incubation with EcoR1 methylase (0.1 µ) was at 37°C for 15 min. Blunt ends were formed by addition of $MgCl_2$ to 10 mM and all deoxynucleotides to 20 µM. After adding 0.25 µg of homogeneous DNA polymerase 1, incubation was at room temperature for 10min. The Sephadex G-100 desalting step was repeated and peak fractions pooled and lyophilized prior to link addition. Eco R1 linkers (12 mer; Collaborativ Research) were phosphorylated before use by the incubation of 3 µg for 1 hr. at 37°C in 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM ATP and 6 U of 4 polynucleotid kinase (Boehringer-Mannheim).

γ -$[^{32}P]$ATP was included so that the reactivity of the phosphorylated linkers could b tested in a trail ligation, the products of which were monitored by electrophoresis in a 10% polyacrylamide gel.

μl of the phosphorylated linkers (0.5 μg) for ligation overnight at 12°C by 0.3 μg of homogeneous T4 ligase. EcoR1 digestion was performed for 1 hr at 37°C in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$ with 1 unit of homogeneous EcoR1. The cDNA was fractionated on a Sephacryl S-100 column (2mm x 15cm in 1 mM Tris-HCl, pH 7.5, 10 μM EDTA). Fractions constituting the leading edge of the cDNA peak were pooled and lyophilized. This material (approximately 30 ng) was annealed with 1 μg of λgt11 DNA which has been digested with EcoR1 and then treated with calf intestine alkaline phosphatase (Boehringer-Mannheim) till the control packaging efficiency was reduced from 10^7 to 10^5 phage per μg. Ligation then proceeded ovemight at 10°C with 0.1 μg of homogeneous T4 ligase. In vitro packaging was performed according to Protocol II of Maniatis, T., Fritsch, E.F. and Sambrook, J., Molecular Cloning, Cold Spring Harbor Laboratory, New York, (1982), using extracts prepared as described therein. Plating a bacterial strain Y1090 indicated 1.3 x 10^6 recombinants, 65% of which contained cDNA inserts as determined by inactivation of β -galactosidase.

For screening, 600,000 members of the amplified library were plated on strain Y1089 at a density of 100,000 per 15 cm plate and grown at 42°C for 3 to 4 hours. Nitrocellulose filters (BA85, Schleicher and Schuell) previously soaked in 10 mM IPTG (isopropyl thiogalactoryranoside) and dried were overlaid on the plates and incubation continued for 2 hr. at 37°C to allow synthesis of the induced β -galactosidase fusion protein. The filters were removed, washed for 3 x 10 minutes in PBS and allowed to dry for 1 hr. at 37°C. The dried filters were incubated in 3% BSA for 4-6 hr. at 37°C. Filters were transferred to a fresh solution of the same composition containing redioiodinated (~10 uCi/μg) monoclonal antibody C219 and incubated at 23°C for 10 hr. Ten minute washes were performed sequentially as follows: two times in PBS, two times 0.1% NP 40 in PBS and three times in PBS. After drying, the filters were autoradiographed. The positives detected in the primary screen were carried through several steps of plaque purification to obtain a homogeneous population of positive phage.

EXAMPLE 2

In order to visualize and determine the size of th fusion protein synthesized as a r sult of insertion of the P-glycoprotein cDNA sequenc into the β-galactosidase gene of λgt11, the recombinant phag was grown lytically on strain Y1089. These c lls w re infected with the λgt11 recombinant phage and plated at low density (about 100 per cells from single colonies were spotted onto two plates and one incubated at 42°C, the other at 32°C. Clones which grew at 32°C but not 42°C were then used to inoculate 100 ml of LB. After growth at 32°C until $OD_{600} = 0.5$, the temperature was rapidly increased to 43°C and incubation continued for 20 minutes. IPTG was added to 10 mM and incubation continued at 37°C for 1 hr. to allow accumulation of induced fusion protein. Cells were harvested by centrifugation at 25°C, resuspended in 1/50 the original volume of PBS and frozen in liquid nitrogen. After thawing, samples were sonicated briefly and electrophoresed on 6.5% polyacylamide gels which were then stained with Coomassie brilliant blue or immunoblotted employing monoclonal antibodies.

With reference to Figure 1, Panel A is aliquots containing 75 μg protein from lysates of Y1089 bacteria infected with λgt11 (lane a) or λCHP-1 (lane b). Molecular weight markers in far left lane were as described previously. Arrows indicate lacZ fusion product of λCHP-1 (top arrow) and uninterrupted β -galactosidase (bottom arrow). Panel B are Western blots of lanes shown in Panel A. Monoclonal antibodies were isolated, and have been classified into three groups on the basis of their ability to recognize three different epitopes on the P-glycoprotein molecule. Three identical sets of blots were probed with representative monoclonal antibodies of Group I (left lanes, C219 probe), Group II (middle lanes, C32 probe) and Group III (right lanes, C494 probe). Lane a, control lysate (λgt11); lane b, lacZ fusion product-containing lysate (λCHP-1). Arrow indicates expected position of lacZ fusion product. The staining of lacZ fusion product with antibodies identifies three independent epitopes.

The plasmid (pCHP-1) or its insert of approximately 600 base pairs can be used as a DNA probe for molecular hybridization analysis of RNA and DNA from drug resistant and drug sensitive cells; for example, in accordance with the DNA probe technique commonly referred to as the Northern and Southern blot tests.

EXAMPLE 3

A comparison of mRNA homologues to th λ CHP-1 phage was undertaken using drug sensitive AuxB1 cells and highly drug r sistant B30 c lls. The results are shown in Figur 2a.

RNA was isolated from monolayer cultures of cells by disruption in guanidinum thiocyanate and CsCl gradient centrifugation. Agarose (1%) gel electrophoresis, transfer to nitrocellulos (BA85, Schleicher and Schuell) and hybridization was according to Thomas. P.S., in Meth. Enzymol, (eds

Press, New York, 100, 255-266 (1983). 15 µg samples of total RNA were dissolved in 1 M deionized glyoxal, 50% DMSO, 10 mM sodium phosphate, pH 6.8 and denatured for 1 hr at 50°C. After chilling, sample buffer (50% glycerol, 10 mM Na phosphate and bromphenol blue) was added and electrophoresis carried out for about 6 hr at 90 V with recirculation of the buffer. For transfer, the gel was placed on Whatman 3MM filter paper soaked in 20 x SCC. Nitrocellulose was wetted with water, placed on the gel and covered with 3mm paper. After blotting for about 10 hr the nitrocellulose was dried under a lamp and baked for 2 hr at 80°C in a vacuum oven.

The insert corresponding to the portion of P-glycoprotein identified from the λgt11 library by monoclonal antibody C219 was prepared for use as a hybridization probe by subcloning into the EcoR1 site of plasmid pUC9. The recombinant plasmid thus obtained was termed pCHP-1 and employed directly as a hybridization probe or as a source of insert to be used as probe.

Prehydridization of the Northern blot was at 42°C for 16 hr in 50% formamide, 5xSCC, 50 mM sodium phosphate, pH 6.5, 250 µg/ml of sonicated salmon sperm DNA, 0.02% BSA, 0.02% Ficoll and 0.02% polyvinyl pyridone. For hybridization 10% dextran sulfate was added. The insert from pCHP-1 labelled by nick translation, was denatured at 100°C for 5 min. before use for hybridization at 42°C for 18 hr. Washing was as follows: 4 times 5 minutes in 2xSSC, 0.1% SDS at 23°C and two times in 0.1 x SSC, 0.1% SDS at 50°C. The washed sheet was autoradiographed to yield the pattern shown in Figure 2a. 28S and 18S indicate the positions of the ribosomal RNAs.

From the Northern blot analysis, it is seen in Figure 2a that a single major messenger RNA component is observed in the B30 cells in greatly increased amounts with a molecular size of just slightly less than 5 kilobases. Both the increased expression and size of the mRNA revealed by the pCHP-1 probe is completely consistent with the pCHP-1 containing sequences complementary to the P-glycoprotein gene. The protein moiety of P-glycoprotein is about 140,000 daltons which requires a mRNA with a minimum of about 3.5 to 4.0 kb of coding region. It appears that the 5' and 3' untranslated sequences increase the size of the P-glycoprotein mRNA to that of the band in Figure 2a which is approximately 5.0 kb.

EXAMPLE 4

As mentioned above, cell lines of increased colchicine resistance hav increased P-glycoprotein expression. pCHP-1 was used as a ship also exists for the amount of P-glycoprotein mRNAs in these lines. Figure 2b shows a slot blot from mRNA prepared from a series of CH clones of increasing drug resistance, namely AuxB1 → CH^RA3 → CH^RB3 → CH^RC5 up to B30 and a revertant clone I10 which was selected from CH^RC5. The slot blot apparatus (similar to that of Schleicher and Schnell) was first soaked in 200 µg/ml denatured salmon sperm DNA in 1M ammonium acetate for 2 hr and rinsed with 1M ammonium acetate. The nitrocellulose onto which RNA was to be blotted was soaked in H_2O and then 20 x SSC as was the underlay of Whatman 3MM. 200 µl of 20 x SSC was loaded onto each slot and allowed to absorb through the underlay. RNA samples (15 µg) prepared as in Figure 2a after denaturing in glyoxal were diluted to 200 µl with, were loaded and washed in with a further 200 µl of 20 x SSC. The apparatus was disassembled and the nitrocellulose, dried, baked and hybridized with pCHP-1 as in Figure 2a.

The clonal lines CH^RA3, CH^RB3, CH^RC5 were derived in sequential selections for colchicine resistance. CH^RB30 was obtained by growing CH^RC5 in increasing colchicine concentrations starting from 5 ug/ml in several steps to 30 µg/ml. Revertant I10 was selected from CH^RC5 in a single step. Relative resistance to colchicine was determined by the amount of drug required to reduce relative colony forming ability to 10% compared with that required for the parental line.

It can be seen that the amount of mRNA revealed by the pCHP-1 probe in these lines is completely consistent with the pCHP-1 sequences encoding a portion of the P-glycoprotein gene. Taken together, all the above data provides evidence that a fragment of the cDNA is cloned for P-glycoprotein.

EXAMPLE 5

Southern blot analysis of genomic DNA from the drug sensitive and highly multidrug resistant CHO cell lines is shown in Figure 3a. Fifteen µg of DNA from each cell was digested to completion with EcoR1 and electrophoreses in 0.6% agarose gels. Transfer to nitrocellulose was performed essentially as described by Southern, E.M. J. Mol. Biol, 98, 503-517 (1975). The filters were prehybridized 9 hr at 40°C in 50% formamide, 5xSCC, 20 mM sodium phosphat , pH 6.5, 200 µg/ml of sonicated salmon testes DNA, 0.1% BSA, 0.1% Ficoll and 0.1% polyvinylpyrrolidone. Hybridization of the filters was in the same solution, with the addition of dextran sulfate to 10%. the probe pCHp-1 was prepared by nick translation with [α-$^{[32]}$p] dCTP to a specific activity of 3 x 10^8 dpm/µg

at 100 °C to a concentration of 10^6 cpm/ml. After hybridizing for 35 hr at 40 °C, the filters were washed 2 times 5 min in 2xSCC, 0.1% SDS at room temperature, 2 times for 30 min in 2xSCC, 0.1% SDS and 0.1% sodium pyrophosphoate at 50 °C, air dried and autoradiographed for 15 hr at -70 °C with intensifying screens. Molecular weights were determined from Hind III-digested λ DNA which was co-electrophoresed. AuxB1 (X12) represents the AuxB1 lane exposed 12 times longer. Band 1 in AuxB1 is under-represented in this experiment. In other experiments, its intensity is similar to band 3 of AuxB1.

From the above, it appears that there are twelve bands of EcoR1 fragments ranging from 1 to approximately 13 kbp in drug sensitive cells. Multiple bands are also observed when other restriction enzymes are used. Since the pCHP-1 probe has no internal EcoR1 sites, and since the insert size is only about 600 bp, the multiple bands observed are indicative of a family of nucleic acid sequences coding for the P-glycoprotein gene in CHO cells. These sequences are amplified in the drug resistant cell line B30. This indicates that the over-expression of P-glycoprotein in this line at the protein and mRNA levels results from gene amplification. The fact that the bands are apparently differentially amplified indicates that P-glycoprotein is encoded by a family of genes. Of interest is the observation of additional amplified bands in B30 DNA not represented by AuxB1 DNA (X12).

EXAMPLE 6

Southern blot analyses of DNAs from other multidrug resistance lines are shown in Figure 3b. DNA was prepared from drug-sensitive (S) or multidrug-resistant (R) lines and Southern blot analysis undertaken under the same conditions as in Figure 3a. Autoradiography was for 7 days except in the resistant mouse line (X0.1) where an exposure for 15 hr is also shown. The resistant mouse cell line was derived from L cells and was selected with colchicine. The resistant human cell line was derived from the human leukemic cell line CCRF-CEM and selected for high resistance to vinblastine.

In mouse cells, a family of bands is obs rved different from that observed in the CHO cells. In the drug-sensitive line, some of the bands are significantly fainter than others. In the human cells, six bands are noted. In the drug-r sistant lines, bands observed in the drug sensitiv cells ar presented in increased copy numbers and in both the human and the mouse systems, differential amplification is observed. In the human system, some of the bands ar greatly enhanced in the whereas others ar not.

These observations demonstrate that P-glycoprotein is encoded by a gene family and, that amplification of members of this family is associated with over-expression of P-glycoprotein.

EXAMPLE 7

Cytological evidence for localization of P-glycoprotein sequences to a homogeneous staining region at the Z4 chromosome of CH^RB30 cells is shown in Figures 4a, 4b and 4c. In Figure 4a, is a representative G-banded CH^RB30 cell demonstrating an HSR on chromosome Z4 (arrow). Such an HSR is not observed in the drug-sensitive AuxB1 line. In Figure 4b, the results of localization of autoradiographic grains to the Z4-HSR region (arrow) following hybridization in situ with ^3H-labelled pCHP-1. In Figure 4c, are examples of grain localization, the Z4-HSR (arrows) are from five different CH^RB30 cells as in Figure 4b.

The results of Figure 4 were generated by G-banding and in situ hybridization analysis performed as described Trent, J.M., Olson, S., Lawn, R.M. Proc. Natl. Acad. Sci. U.S.A. 79, 7809-7813 (1982) and Schwab, M., Alitalo, J., Klempnauer, K-H., Varmus, H.E., Bishop, M.J., Gilbert F., Brodeur, G., Goldstein, M. and Trent, J.M. Nature 305, 245-248 (1983). The most consistent and characteristic chromosomal alteration between the parental line AuxB1 and highly colchicine-resistant CH^RB30 line is the presence of an HSR on the long arm of the Z4 chromosome as shown in Figure 4a. In the in situ hybridization procedure, cells were hybridized for 15.5 hr at a final DNA concentration of 0.65 μg ml, followed by exposure for 7 days prior to autoradiographic development as in Figures 4b and 4c. The autoradiographic analysis was performed with unbanded chromosomes; however, the size and arm length ratio of the Z4-HSR allowed it to be unequivocally identified in CH^RB30 cells.

According to this invention, over-expression of P-glycoprotein genes in the multidrug resistance lines is accompanied by gene amplification. The recent observation that over-expression of P-glycoprotein does occur in advanced ovarian tumour cells suggests that this mechanism is also operative in human malignancies. Sinc gene amplification featuring HSRs is commonly observed in malignant diseases, it is possible that multidrug resistance mutations may be relatively common and that such mutations may limit successful combination chemotherapy.

The selection of cells having th P-glycoprotein in their membranes r sulting in the isolation of a nucleic acid sequence coding for a polypeptid portion of the P-glycoprotein, provides a variety of

the cDNA is of about 5.0 kb in siz and the amount is in direct relation to the amounts of the protein and the degree of multidrug resistanc in the cell. The cDNA may be used as a DNA probe to recognize P-glycoprotein sequences in human and animal cells and determine amplification of these genes in multidrug resistance. P-glycoprotein genes constitute a multi-gene family, the members of which may be differentially amplified.

Some of the gene sequences for the P-glycoprotein are transferrable to drug sensitive cells when resistance is transferred by transfection with genomic DNA from resistant cells.

The complementary DNA can be used to identify and isolate functional gene sequences of both the cDNA and genomic DNA. Functionality can be monitored by the quantitation of drug resistance after transfection with the cloned sequences. In addition to the development of the primary drug resistance function, these transfection experiments can be used to study the regulation of plasma membrane permeability and transport of large and small molecules. The cDNA probe can be used in the diagnosis of the development of multidrug resistance in cancer patients. This detection capability provides a basis for improved chemotherapy regimes. Furthermore, the system can be used in the detection of tumour cells with amplified P-glycoprotein genes to enable the targeting of chemotherapeutic agents directly to those tumour cells.

Claims

1. A purified DNA molecule of approximately 600 bp characterized in that said DNA molecule codes for a polypeptide portion of approximately 200 amino acids of a P-glycoprotein which has a total molecular weight of approximately 170,000 daltons and an isoelectric point of 7.4, said DNA molecule including DNA molecules pCHp-1 and pCHp-2 having respectively ATCC accessions numbers 39839 and 53234, said P-glycoprotein being a major determinant in multidrug resistance of living cells.

2. A purified DNA molecule according to claim 1, wherein said DNA molecule belongs to a gene of a gene family each of which codes for the P-glycoprotein.

3. A purified DNA molecule according to claim 2, wherein said gene family is a multi-gene family.

4. A purified DNA molecule according to claim 1, wherein said DNA is in the form of cDNA.

5. A purified DNA molecule according to claim 1, wherein said DNA is in the form of mRNA.

6. A purified DNA molecule according to claim 1, wherein said DNA is in recombinant form with a bacterial phage.

7. A purified DNA molecule according to claim 1, wherein said DNA is in recombinant form with a plasmid.

8. A purified DNA molecule according to claim 6 or 7, wherein said DNA fragment is in the form of cDNA.

9. A purified DNA molecule according to claim 5, wherein said mRNA is of approximately 5 kb and has a coding region for said P-glycoprotein of approximately 3.5 to 4.0 kb which codes for a fusion protein of approximately 140,000 daltons for said P-glycoprotein which comprises a β-galactosidase portion of approximately 116,600 daltons and a C-terminal peptide portion of 23,400 daltons.

10. A purified DNA molecule according to claim 6, wherein said bacterial phage is adapted for use as a DNA probe.

11. A purified DNA molecule according to claim 7, wherein said plasmid is adapted for use as a DNA probe.

12. A purified DNA molecule according to claim 8, wherein said cDNA is adapted for use as a DNA probe.

13. A purified DNA molecule according to any one of claims 1 to 12, wherein said DNA molecule hybridizes specifically to a gene encoding P-glycoprotein and mRNA transcripts of said gene of Chinese Hamster Ovary cells, mouse cells and human cells.

14. A purified DNA molecule according to any one of claims 10, 11 or 12, wherein said DNA probe is labelled with a detectable marker.

15. A host c ll comprising a plasmid according to claim 7.

16. A microorganism comprising a plasmid according to claim 7.

17. An E. coli microorganism according to claim 16 having ATCC accession No. 39839 or 53234 and comprising said plasmid of claim 7.

18. A method of producing a polypeptide portion of a P-glycoprotein having a total molecular weight of approximately 170,000 daltons characterized in culturing a microorganism according to claims 15, 16 or 17 in an aqueous nutrient medium and isolating said polypeptide.

19. A method of isolating in a plasmid said cDNA according to claim 4, wherein a cDNA library is constructed using a known bacterial phage expression vector and mRNA obtained from highly drug-resistant cell lines which over-express said P-glycoprotein, identifying recombinant phage vectors which express a fusion protein antigenic to an antibody which is specific to said P-glycoprotein, forming a recombinant plasmid from identified phages, expressing said plasmid in a compatible living cell to produce a polypeptide portion, and confirming by use of said antibody that said polypeptide is said portion of said P-glycoprotein.

20. A method according to claim 19, wherein said bacterial phage is λgt11.

21. A method according to claim 19, wherein said plasmid is pUC9.

Patentansprüche

1. Gereinigtes DNA-Molekül mit etwa 600 bp, **dadurch gekennzeichnet**, daß das DNA-Molekül aus einem Anteil von Polypeptiden von etwa 200 Aminosäuren eines P-Glykoproteins kodiert ist, das ein Gesamt-Molekulargewicht von etwa 170.000 Dalton und einen isoelektrischen Punkt von 7,4 hat, daß das DNA-Molekül DNA-Moleküle pCHp-1 und pCHp2-2 enthält, die ATCC-Zulassungszahlen 39839 bzw. 53234 haben, und daß das P-Glykoprotein eine Haupt-Determinante in einer vielfachen Drogen-Resistenz lebender Zellen ist.

2. Gereinigtes DNA-Molekül nach Anspruch 1, in dem das DNA-Molkekül zu einem Gen oder einer Gen-Familie gehört, von der jede nach dem P-Glykoprotein kodiert ist.

3. Gereinigtes DNA-Molekül nach Anspruch 2, in dem die Gen-Famili eine Multi-Gen-Familie ist.

4. Gereinigtes DNA-Molekül nach Anspruch 1, in dem das DNA die Form von cDNA hat.

5. Gereinigtes DNA-Molekül nach Anspruch 1, in

6. Gereinigtes DNA-Molekül nach Anspruch 1, in dem das DNA sich in einer rekombinanten Form mit einer Bakteriophage befindet.

7. Gereinigtes DNA-Molekül nach Anspruch 1, in dem das DNA sich in einer rekombinierten Form mit einem Plasmid befindet.

8. Gereinigtes DNA-Molekül nach Anspruch 6 oder 7, in dem das DNA-Fragment sich in der Form von cDNA befindet.

9. Gereinigtes DNA-Molekül nach Anspruch 5, in dem das mRNA etwa 5 kb beträgt und einen Kodierbereich für das P-Glykoprotein von etwa 3,5 bis 4,0 kb hat, der für eine Protein-Fusion von etwa 140.000 Dalton für das P-Glykoprotein kodiert ist, das einen β-Laktoglukose-Anteil von etwa 116.600 Dalton und einen C-Terminal Peptide-Anteil von 23.400 Dalton enthält.

10. Gereinigtes DNA-Molekül nach Anspruch 6, in dem die Bakteriophage für eine Anwendung als DNA-Probe vorgesehen ist.

11. Gereinigtes DNA-Molekül nach Anspruch 7, in dem das Plasmid für eine Anwendung als DNA-Probe vorgesehen ist.

12. Gereinigtes DNA-Molekül nach Anspruch 8, in dem das cDNA für eine Anwendung als DNA-Probe vorgesehen ist.

13. Gereinigtes DNA-Molekül nach einem der Ansprüche 1 bis 12, in dem das DNA-Molekül spezifisch mit einem Gen gekreuzt wird, das P-Glykoprotein und mRNA-Umsetzungen dieses Gens von Eierstockzellen chinesischer Hamster, Mäusezellen und menschlichen Zellen enthält.

14. Gereinigtes DNA-Molekül nach einem der Ansprüche 10, 11, oder 12, in dem die DNA-Probe mit einer detektierbaren Markierung etikettiert ist.

15. Gast-Zelle, enthaltend ein Plasmid nach Anspruch 7.

16. Mikroorganismus, enthaltend ein Plasmid nach Anspruch 7.

17. E. Coli-Mikroorganismus nach Anspruch 16 mit der ATCC-Zulassung Nr. 39839 oder 53234 und nthaltend das Plasmid nach Anspruch 7.

18. Verfahren zur Herstellung eines polypeptiden

Molekulargewicht von etwa 170.000 Dalton, **dadurch gekennzeichnet**, daß ein Mikroorganismus nach Anspruch 15, 16 oder 17 in einem wässerigen Ernährungsstoff gezüchtet und das Polypeptid isoliert wird.

19. Verfahren zur Isolierung des cDNA nach Anspruch 4 in einem Plasmid, in dem ein cDNA-Verzeichnis gebildet wird, das einen bekannten Baktieriophage-Ausdruck-Vektor und mRNA enthält, die von hoch-drogenresistenten, das P-Glykoprotein überausdrückenden Zellinien gewonnen sind, daß die rekombinanten Phage-Vektoren identifiziert werden, die eine zu einem Antikörper antigene Proteinfusion ausdrücken, die spezifisch zu dem P-Glykoprotein ist, daß ein rekombinantes Plasmid aus den identifizierten Phagen gebildet wird, daß das Plasmid in einer verträglichen lebenden Zelle ausgedrückt wird, um einen polypeptiden Anteil zu erzeugen, und daß durch Anwendung des Antikörpers sichergestellt wird, daß das Polypeptid der Teil des P-Glykoproteins ist.

20. Verfahren nach Anspruch 19, bei dem die Bekteriophage λgt11 ist.

21. Verfahren nach Anspruch 19, bei dem das Plasmid pUC9 ist.

Revendications

1. Molécule d'ADN purifiée d'environ 600 paires de bases, caractérisée en ce que cette molécule d'ADN code pour une portion polypeptide d'environ 200 acides aminés d'une P-glycoprotéine qui a un poids moléculaire total d'environ 170 000 daltons et un point isoélectrique de 7,4, cette molécule d'ADN contenant des molécules d'ADN pCHP-1 et pCHP-2 ayant respectivement des numéros de référence ATCC 39839 et 53234, cette P-glycoprotéine étant un déterminant principal dans la résistance à plusieurs médicaments des cellules vivantes.

2. Molécule d'ADN purifiée selon la revendication 1, dans laquelle cette molécule d'ADN appartient à un gène d'une famille de gènes dont chacun code pour la P-glycoprotéin .

3. Molécule d'ADN purifiée selon la revendication 2, dans laquelle cette famill de gènes est une famille de gènes multiples.

4. Molécule d'ADN purifiée selon la revendication 1, dans laquelle cet ADN est sous la forme d'ADNc.

5. Molécule d'ADN purifiée selon la revendication 1, dans laquelle cet ADN est sous la forme d'ARNm.

6. Molécule d'ADN purifiée selon la revendication 1, dans laquelle cet ADN est dans une forme recombinée avec un phage bactérien.

7. Molécule d'ADN purifiée selon la revendication 1, dans laquelle cet ADN est sous forme recombinée avec un plasmide.

8. Molécule d'ADN purifiée selon la revendication 6 ou la revendication 7, dans laquelle ce fragment d'ADN est sous la forme d'ADNc.

9. Molécule d'ADN purifiée selon la revendication 5, dans laquelle cet ARNm a une longueur d'environ 5 kilobases et a une région codante pour la P-glycoprotéine d'environ 3,5 à 4,0 kilobases, qui code pour une protéine de fusion d'environ 140 000 daltons pour ladite P-glycoprotéine, qui comprend une portion β-galactosidase d'environ 116 600 daltons et une portion peptide en C-terminal de 23 400 daltons.

10. Molécule d'ARN purifiée selon la revendication 6, dans laquelle le phage bactérien est adapté pour être utilisé comme sonde ADN.

11. Molécule d'ADN purifiée selon la revendication 7, dans laquelle le plasmide est adapté pour être utilisé comme sonde ADN.

12. Molécule d'ADN purifiée selon la revendication 8, dans laquelle cet ADNc est adapté pour être utilisé comme sonde ADN.

13. Molécule d'ADN purifiée selon l'une des revendications 1 à 12, dans laquelle cette molécule d'ADN s'hybride spécifiquement à un gène codant la P-glycoprotéine et a des transcripts d'ARNm de ce gène de cellules d'ovaires de hamsters chinois, de cellules de souris et de cellules humaines.

14. Molécule d'ADN purifiée selon l'un des revendications 10, 11 ou 12, dans laquell cette sonde ADN est marquée avec un marqueur détectable.

15. Cellule hôte comprenant un plasmide selon la revendication 7.

16. Micro-organisme comprenant un plasmide selon la revendication 7.

17. Micro-organisme E. coli selon la revendication 16, ayant un numéro de référence ATCC No 39839 ou 53234 et comprenant ce plasmide de la revendication 7.

18. Méthode pour produire une portion polypeptide d'une P-glycoprotéine ayant un poids moléculaire total d'environ 170 000 daltons, caractérisée en ce qu'on cultive un micro-organisme selon l'une des revendications 15, 16 ou 17 dans un milieu nutritif aqueux et qu'on isole ce polypeptide.

19. Méthode pour isoler dans un plasmide l'ADNc selon la revendication 4, dans laquelle on construit une banque d'ADNc en utilisant un vecteur d'expression de phages bactériens connu et un ARNm obtenu de lignées de cellules très résistantes aux médicaments, qui surexpriment ladite P-glycoprotéine, en identifiant des vecteurs phages recombinés qui expriment une protéine de fusion antigénique à un anticorps spécifique de ladite P-glycoprotéine, en formant un plasmide recombiné à partir de phages identifiés, en exprimant ce plasmide dans une cellule vivante compatible pour produire une portion polypeptide et en confirmant par l'utilisation de cet anticorps que le polypeptide est ladite portion de la P-glycoprotéine.

20. Méthode selon la revendication 19, dans laquelle le phage bactérien est λgt11.

21. Méthode selon la revendication 19, dans laquelle le plasmide est pUC9.

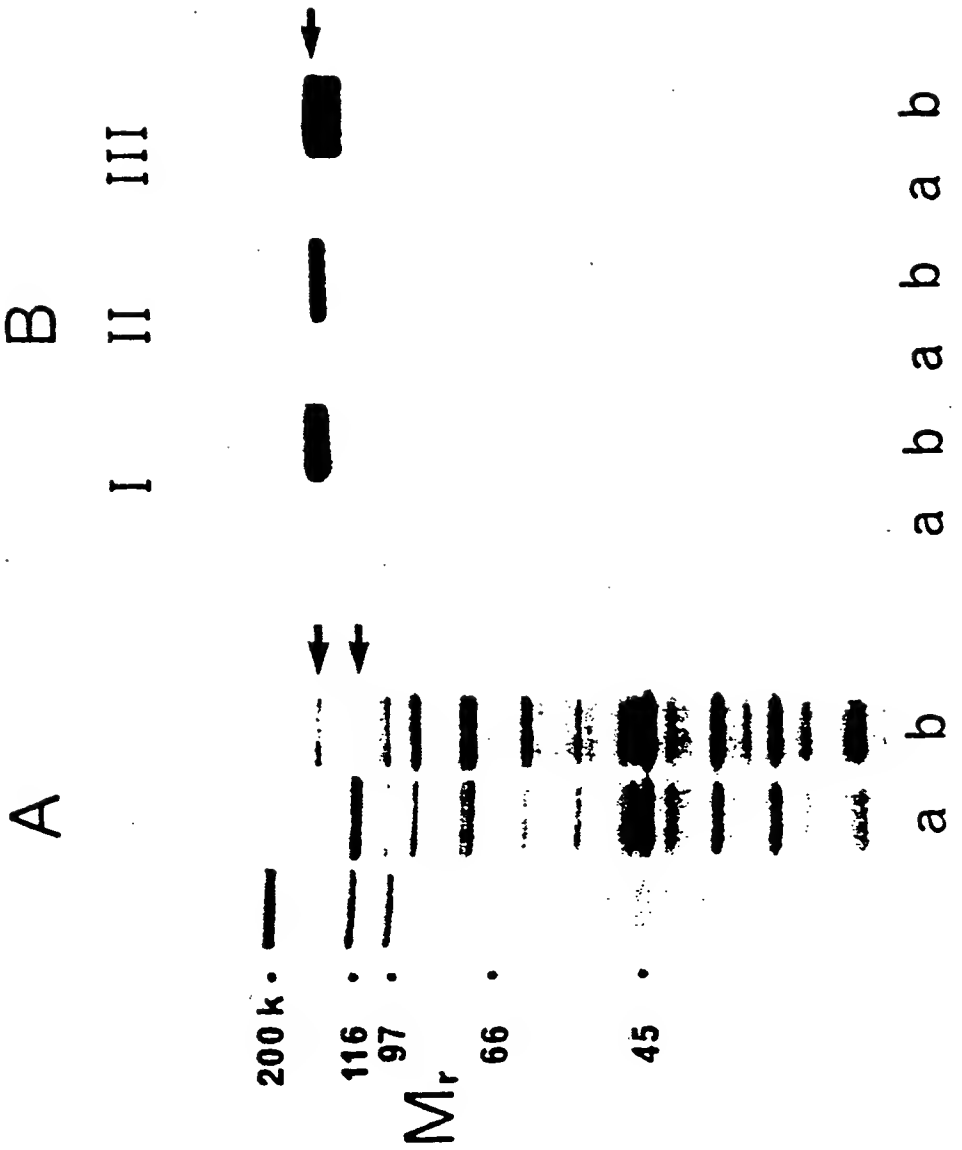

FIG.1.

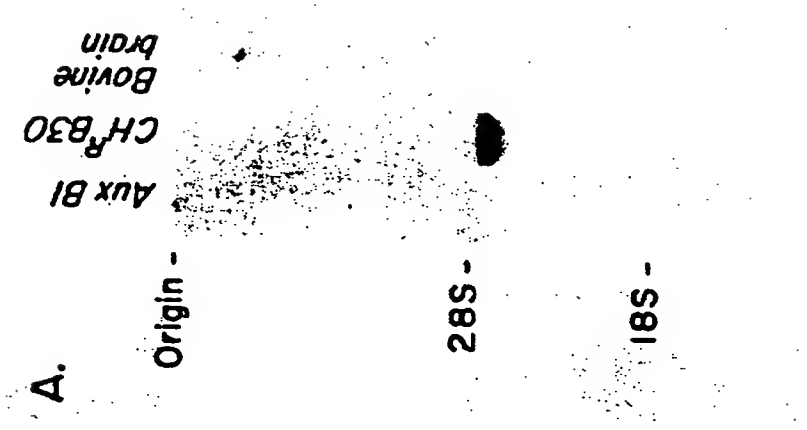

FIG. 2A.

B.

RNA: Slot - blot	Relative Resistance
- AuxBI	1
- $CH^R A3$	6
- $CH^R B3$	20
- $CH^R C5$	170
- $CH^R B30$	>>600
- 110	3

FIG. 2B.

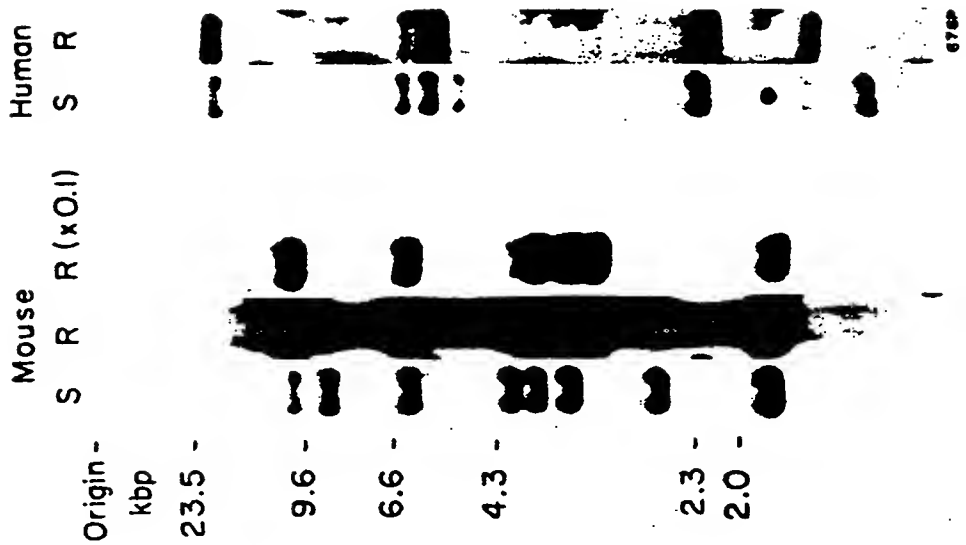
Human
S R
Mouse
S R R (xO.1)
Origin -
kbp
23.5 -
9.6 -
6.6 -
4.3 -
2.3 -
2.0 -

FIG.3B.

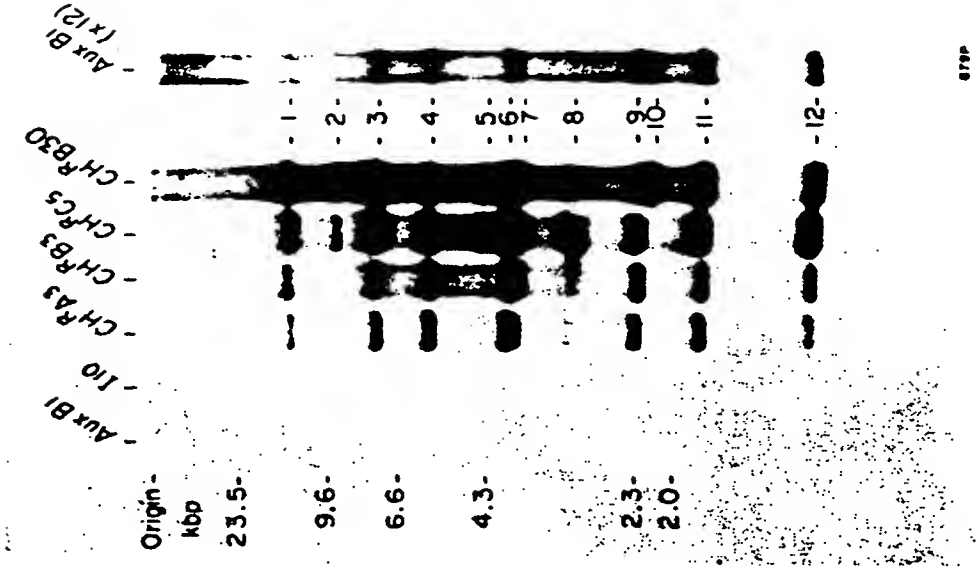
Aux BI (x 12)
CH RB30
CH RC3
CH RB3
CH RA3
I10
Aux BI
Origin -
kbp
23.5 -
9.6 -
6.6 -
4.3 -
2.3 -
2.0 -
1
2
3
4
5
6
7
8
9
10
11
12

FIG.3A.

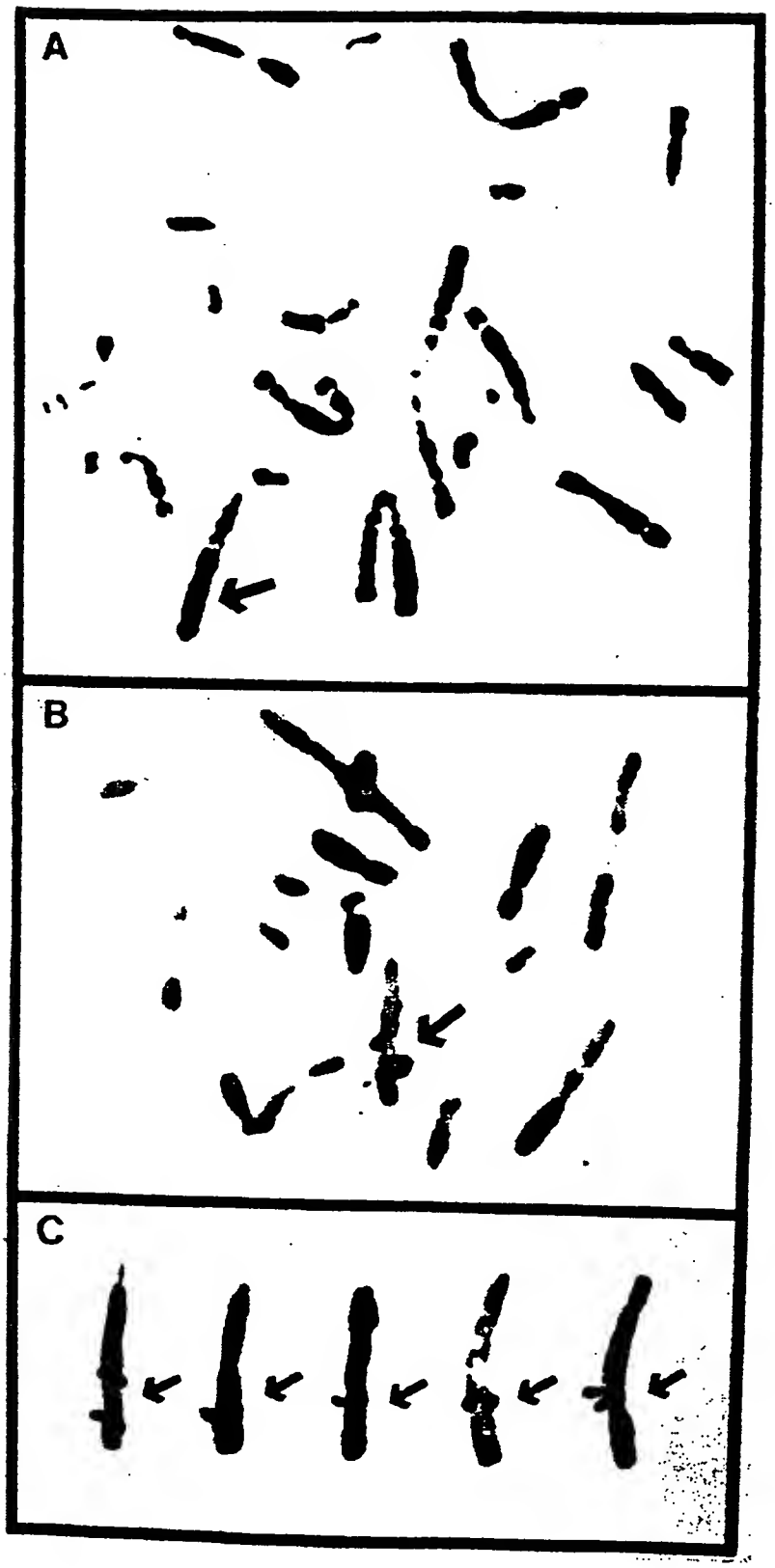

FIG.4A.

FIG.4B.

FIG.4C.